Fungal diversity during fermentation correlates with thiol concentrations in wine

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Short running title (50 characters): Fungal diversity correlates with wine thiol levels

Abstract

Background and Aims: Agricultural products deriving from the same genotypic clone often have different physical and sensorial properties that influences their overall quality and value. Microbes may play key roles throughout the production of many crops, affecting plant and fruit health and modifying plant materials to produce socially and economically important commodities. Following this idea, we investigated whether fungal diversity both prior to and during fermentation was correlated with the concentration of three volatile thiols important to Sauvignon blanc aroma and flavour.

Methods and Results: We used molecular and metagenomics approaches to quantify yeast populations and GC-MS to quantify thiols and analysed these using random forest statistical approaches. The species of *Saccharomyces* yeasts present at the end of fermentation significantly correlated with the concentration of 4MMP, while a number of other fungal species present in the must, that are known to be associated with vine and fruit health, also correlated with thiol concentrations.

Conclusions: These data highlight the relationship between the presence of *S. uvarum* and the production of 4MMP, while some members of the fungal community correlate with thiol concentrations generally. Thus, components of the fungal community may potentially affect the accumulation of odourless precursors in grape via pathogenic effects during fruit ripening, but further research is required to confirm these speculations.

Significance of the study: This recapitulates the need for a better understanding of the interactions between microbial populations and agricultural products, and has implications for the management of fungal diversity and disease in these systems.

Key words: Fungi metagenomics, Saccharomyces, Sauvignon blanc, Thiols

INTRODUCTION

Microbes play key roles in the production of quality agricultural goods, affecting plant and fruit health and converting plant materials into socially and economically important commodities (Whipps 2001, Fleet 2006, Peiffer et al. 2013, Philippot et al. 2013). During the processing of plant materials such as grapes for winemaking, different species and strains of yeast are known to produce different concentrations of volatile compounds that contribute different sensorial properties to the final products (Howell et al. 2004, Viana et al. 2008, Anfang et al. 2009, Zott et al. 2011). In wine, many of these yeast-derived aromas and flavours produced during fermentation result from the conversion of odourless precursors in the grape must (Darriet et al. 1995, Tominaga et al. 1998a, Swiegers and Pretorius 2005, Dubourdieu et al. 2006). Pathogenic fungi present on vines and grapes may also potentially alter concentrations of odourless precursors in the grapes of infected vines and bunches (Thibon et al. 2009, Thibon et al. 2011, Barata et al. 2012) and thus may potentially affect the final flavour and aroma of a wine. Volatile thiols are one class of compounds produced from aroma-less precursors by microbes, and these tend to positively contribute to wine styles (refs). Here we investigate the relationship between volatile thiol concentrations in wine and fungal species (including yeast) diversity in grape juice and during fermentation to reveal the effects of microbial species diversity on one sensory aspect of wine.

Spontaneous ferments of grape juice to wine are completed by a succession of indigenous yeasts that naturally occur on grapes which are transferred to the grape must (Pretorius 2000, Xufre et al. 2006). During the early stages of a spontaneous ferment, a diversity of yeast species is observed

with *Saccharomyces* species being very rare (Pretorius 2000, Xufre et al. 2006, Goddard 2008). As the ferment progresses, *Saccharomyces* species outcompete other microbes by engineering the ecosystem through the preferential fermentation of sugars to create a toxic hot anaerobic alcoholic environment (Goddard 2008). Through this fermentative process, *Saccharomyces* species also produce a wide range of metabolites that have a positive influence on wine sensory attributes including volatile thiols (Lambrechts and Pretorius 2000, Swiegers et al. 2006, Swiegers et al. 2009). Typically wine fermentation is performed by *S. cerevisiae*, although the presence of *S. uvarum* has also been widely reported (Torriani et al. 1999, Naumov et al. 2000, Demuyter et al. 2004, Masneuf-Pomarède et al. 2010, Zhang et al. 2010). Studies show these two species of *Saccharomyces* produce different levels of volatile compounds during wine fermentation (Murat et al. 2001, Masneuf et al. 2002, Dubourdieu et al. 2006, Masneuf-Pomarède et al. 2010). Other species of the *Saccharomyces* sensu stricto species complex are rarely reported associated with vineyards and wine ferments (Naumova et al. 2005, Sicard and Legras 2011).

Non-*Saccharomyces* species present in the early stages of fermentation have also been shown to contribute desirable sensory properties and complexity to the wine (Romano et al. 2003, Clemente-Jimenez et al. 2005, Ciani et al. 2006, Hernández-Orte et al. 2008, Anfang et al. 2009, Comitini et al. 2011, Gobbi et al. 2013), although negative effects have also been reported (Comitini et al. 2011). Much work has been done to evaluate the contribution of particular non-*Saccharomyces* yeasts to wine composition, often using co-inoculation trials with *S. cerevisiae*, and different species have been shown to contribute different attributes (Ciani and Maccarelli 1998, Romano et al. 2003a, Clemente-Jimenez et al. 2005, Hernández-Orte et al. 2008, Ciani et al. 2010, Comitini et al. 2011). While non-*Saccharomyces* yeasts appear to play a role in the final flavour and aroma of a wine,

Saccharomyces species are required to complete the ferment with different species and strains interacting to produce unique flavour and aroma profiles in the finished wine (Howell et al. 2006, Anfang et al. 2009, Sadoudi et al. 2012). Both fungal communities and *S. cerevisiae* populations have been shown to vary with geographic region (Bokulich et al. 2014, Taylor et al. 2014, Knight and Goddard 2015) affording the potential for microbes to contribute to regionally distinct wine phenotypes (Knight et al. 2015, Bokulich et al. 2016).

Sauvignon blanc is a major contributor to the New Zealand (NZ) wine industry comprising 86% of wine exports (New Zealand Winegrowers 2016). The volatile thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercapto-hexan-1-ol (3MH) and 3-mercapto-hexan-1-ol acetate (3MHA) are important in Sauvignon blanc aroma and flavour and are typically described as having box tree, passion fruit, black current bud, broom, grapefruit and guava characteristics (Dubourdieu et al. 2006, Swiegers et al. 2009, Coetzee and du Toit 2012). These compounds are highly potent with low sensory detection thresholds and are made by yeast during fermentation from odourless precursors in the grape must (Darriet et al. 1995, Tominaga et al. 1998a, Dubourdieu et al. 2006, Coetzee and du Toit 2012).

Here we spontaneously ferment commercially derived *Vitis vinifera* var. Sauvignon blanc grape juice sourced from different geographic regions in NZ and correlate the resulting thiol concentrations with fungal diversity in these juices and ferments. Fungi present at the start and *Saccharomyces* species present at the end of fermentation were quantified as well as the final concentration of 3MH, 3MHA and 4MMP.

MATERIALS AND METHODS

Sample collection and fermentation

Sauvignon blanc juice was collected from 37 vineyards across NZ, comprising six vineyards from each of Hawke's Bay, Martinborough, Nelson, the Awatere Valley, Central Otago and the Wairau Valley (an extra sample derived from the Wairau) and samples were taken from the tank after pressing (see Supplementary Figure S1 for locations). These samples were couriered on ice to the University of Auckland. A 50 mL sample of each juice was centrifuged at 3000 rpm for 5 minutes to pellet microbes. The supernatant was discarded and the cells frozen at -20 °C for further community sequencing analyses detailed below. Spontaneous ferments of each juice were performed at 15 °C in 10 L volumes. Sauvignon blanc is typically fermented at lower temperatures in NZ and thus 15 °C was chosen to reflect industry practices. As spontaneous ferments progress, the yeast community becomes dominated by *Saccharomyces* species, most commonly reported as *S. cerevisiae* and *S. uvarum* (Torriani et al. 1999, Naumov et al. 2000, Demuyter et al. 2004, Masneuf-Pomarède et al. 2010, Zhang et al. 2010). However, data shows the formation of the thiols 3MH and 3MHA occur early in the ferment (Harsch et al. 2013). Thus after 21 days, 50 mL samples were taken to characterise the *Saccharomyces* populations and thiol concentrations of the wine. These sampled were again centrifuged at 3000 rpm for 5 minutes and frozen at -20 °C for later chemical analyses.

Fungal community analysis of the juice

The fungal community composition in the initial juice samples was quantified using Roche 454 next generation sequencing technology (Margulies et al. 2005). Total DNA was extracted from the juice samples using the Zymo Research Soil Microbe DNA MiniPrep^{TM} kit. A 600 bp fragment of the

D1/D2 26S ribosomal RNA locus, known to provide good signal for fungal community differentiation (Taylor et al. 2014), was amplified using the fungal specific primers NL1 and NL4 (Kurtzman and Robnett 2003). Distinct multiplex identifiers were added to the primers to bioinformatically distinguish between samples. AmpureXP beads were used to clean the PCR products and remove primer dimers with the final quality confirmed using Agilent DNA1000 chips. The samples were multiplexed and uni-directionally sequenced using a Roche 454 GS Junior sequencer at the University of Auckland.

Post-processing of DNA sequencing data was performed using Mothur version 1.30 (Schloss et al. 2009). Low quality and erroneous sequences were removed starting with primers, low quality reads and reads smaller than 200 bp. Subsequently homopolymer errors as identified using the PyroNoise algorithm (Quince et al. 2009) and finally PCR chimeras identified using the UCHIME algorithm (Edgar et al. 2011) were removed. Individual sample identifiers were assigned to the remaining reads and the data was merged for further analyses. To further account for potential error in the dataset, unique sequences were compared to a fungal reference database and those not assigned to Fungi were removed. The remaining sequences were clustered into groups or operational taxonomic units (OTUs) sharing more than 98 % identity. Multiple species of Ascomycota and Basidiomycota (Fungi) have empirically been shown to differ by less than 2 % at the 26S rDNA gene (Kurtzman and Robnett 2003, Romanelli et al. 2010) thus these groups are considered to approximate species. To taxonomically identify each OTU, the 'classify.seqs' command in Mothur was used. This script selects a representative sequence for each OTU (i.e. a sequence that had a minimum distance to other sequences in the same cluster). These sequences were compared to a

fungal taxonomic database and classified to all levels including and above genus level. Consensus sequences with less than a 70 % match at any taxonomic level were listed as unclassified.

As further quality control, any OTU that had less than five reads total, or were present in only one sample, were conservatively removed. The raw counts of reads assigned to each OTU were converted into proportions for each sample to standardise for the variation in reads per sample (McMurdie and Holmes 2014). The sequence data is available under accession number (to be advised).

Saccharomyces species analysis at the end of ferment

Yeast from the 21st day of spontaneous ferments were identified using culture based methods and molecular identification, but not metagenomics analyses as *Saccharomyces* species are expected to dominate the community at this time point (Goddard 2008). Samples were plated in serial dilutions on YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose with 50 µg/ml chloramphenicol to retard bacterial growth) and incubated at 28°C for two days. Colonies observed on these plates were all round, smooth and cream in colour, typical of *Saccharomcyes* species. Thus 94 individual colonies from each sample were isolated for molecular identification. Genomic DNA was extracted with a 1.25 mg/mL zymolyase solution dissolved in 1.2 M Sorbitol and 0.1 M KH_2PO_4 at pH 7.2 and treated with EMA to bind unwanted DNA fragments (Rueckert and Morgan 2007). *S. cerevisiae* and *S. uvarum* are the most commonly reported *Saccharomyces* species from wine fermentation, while other members of the *sensu stricto* complex are more commonly associated with natural environments (Naumova et al. 2005, Sicard and Legras 2011). A multiplex PCR was performed with primers that can identify *S. cerevisiae*, *S. uvarum* and *S. pastorianus* (de Melo Pereira et al. 2010).

Of the identified isolates, the proportion of *S. uvarum* in each sample was calculated. It is important to note that since only two *Saccharomyces* species were identified in the ferment samples, the inverse of the proportion of *S. uvarum* is the proportion of *S. cerevisiae*.

Thiol analysis

The day-21 wine samples (50 mL) were sent to Hill Laboratories Limited, Hamilton, NZ for chemical analysis. Quantitative analyses were performed for the volatile thiols 3-mercaptohexanol (3MH), 3-mercaptohexyl acetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP). Chemical compounds were extracted from the wine samples using Solid Phase Micro Extraction (SPME) and quantified in extracts using Gas Chromatography coupled with Mass Spectrometry (GCMS).

Statistical analysis

To investigate the relationship between the samples' microbial community (the explanatory variables) and final thiol concentrations (the response variables) we used a combination of approaches. Thiol concentrations were log transformed to account for exponential behaviour of measurements. Regional variation of thiol potential was identified as a potential confounder (Lund et al. 2009, Benkwitz et al. 2012), and thus the origin of juices was included in models to account for this variation. Random forest analysis (Breiman 2001, Cutler et al. 2007) was employed to identify the covariates most important in explaining the variation in thiol concentrations. A random forest analysis generates bootstrapped regression trees based on the explanatory variables, and uses the averages of these trees to estimate the relative importance of each of the variables in explaining the response (Cutler et al. 2007). To assess the importance of a variable the average prediction error is used which assesses how useful a variable is to determine the value of the

response. It is measured by the mean squared error and is computed using a permutation approach (Hastie et al. 2009). The importance of all explanatory variables was ranked and the top ranked variables were further investigated using conditional inference trees (Horthorn et al. 2006). For a partition to be generated in a conditional inference tree, a statistically significant difference is required, minimising bias and over-fitting (Horthorn et al. 2006). All analyses were performed in R version 3.2.1 using the packages 'randomForest' (Liaw and Wiener 2002) and 'party' (Horthorn et al. 2006).

To further investigate the effect of the *Saccharomyces* species driving the ferment, Pearson's product-moment correlations were calculated between the proportions of *S. uvarum* as a total of all *Saccharomyces* isolates found in the day-21 wine samples and the thiol concentrations in these samples.

RESULTS

The 454-sequencing of the initial juice samples resulted in a total of 29 253 quality reads after processing. In 11 samples, the proportion of OTU002 was high (Supplementary Dataset S1). This OTU was identified as the genus *Saccharomyces* which should be rare prior to fermentation (Goddard 2008). A high proportion of *Saccharomyces* suggests fermentation had started in these samples, possibly due to large proportion of damaged berries (Mortimer and Polsinelli 1999) or variance in transit times to the laboratory. We do not have any juice composition data to determine whether the high proportion of *Saccharomyces* in these samples was due to damaged fruit or the start of fermentation, and thus we conservatively removed samples for which the *Saccharomyces* abundance exceeded 10 %, and this reduced the dataset to 26 samples. After removing OTUs that

had less than five reads and were only present in one sample (as explained above), the number of quality reads for each sample ranged from 149 – 1372 and 88 OTUs were defined in total (Supplementary Dataset S2).

At the end of fermentation, two species of *Saccharomyces* were identified: *S. cerevisiae* and *S. uvarum*. Isolates from some samples were unable to be identified due to PCR failure and were scored as unknown (Supplementary Dataset S1). Failed samples were randomly distributed among samples, and over 70 isolates from each sample were positively identified, and used to calculate the proportion of each species. *S. cerevisiae* and *S. uvarum* varied in proportion across the day-21 samples: eight samples reported no *S. uvarum*, and one was entirely *S. uvarum* (see supplemental dataset 1).

A wide range of concentrations were observed for all three volatile thiols quantified (Supplementary Dataset S1). One sample from Martinborough (MARLI) reported concentrations of both 3MH and 3MHA below the detection threshold of the analysis (10 ng/L), and 4MMP was very low at just 16 ng/L. As two of the three response variables were unable to be quantified, this sample was removed from further analyses, resuling in a total of 25 samples. Of these remaining samples, the highest concentrations of 3MH was 10,100 ng/L, 3MHA with 2,745 ng/L, and 4MMP with 291 ng/L (Supplementary Dataset S2). All thiols exhibited a skewed distribution with many smaller values and a long tail of few, larger values. We therefore used the log of these concentrations for analysis to avoid larger values biasing the analyses. 4MMP was unable to be quantified in four samples as it was below the 10 ng/L detection threshold of the analysis. For analytical purposes, the concentration for these samples was imputed as a random number

between zero and ten (the detection limit of the assay). This approach permits the inclusion of these samples in analysis while preventing undue influence on the model. The final dataset can be seen in Supplementary Dataset S2.

Yeast modulate the balance between 3MH and 3MHA through the production of an alcohol acetyltransferase that converts 3MH to 3MHA, and an esterase enzyme that reverses this reaction (Swiegers et al. 2006). These two volatile thiols thus exist in an equilibrium and should be correlated with one another as previously been reported by Masneuf-Pomarède et al. (2006). Pearson's product-moment correlation coefficient shows 3MH and 3MHA as significantly positively correlated ($r = 0.72$, $t_{34} = 6.10$, $P = 6.37 \times 10^{-7}$). Since the concentration of these compounds is not independent, we analysed the molar sum of 3MH and 3MHA to investigate their total production as well as the ratio between the two to test if yeast species affects their equilibrium.

The effects of species diversity on the concentration of 4MMP

Random forest analysis revealed 44 % of the observed variation in 4MMP concentration may be explained by region, proportion of *S. uvarum* in the day-21 samples, and the proportion of the 88 OTUs identified in the juice. The proportion of *S. uvarum* at the end of ferment and the region the juice was sourced from stand out as the two most important variables explaining the concentration of 4MMP in the day-21 wine samples (Figure 1). Otu0011 and Otu0046 were ranked next with similar importance scores, and the importance of subsequent variables drops away (Figure 1). Taxonomic identification suggest that Otu0011 corresponds to the genus *Alternaria* and Otu0046 corresponds to the genus *Penicillium*. Partial dependence plots indicate that 4MMP concentration is highest in the Wairau and Awatere Valleys, which are located in the Marlborough region, and in

Martinborough (Figure 2). The relationship between the proportion of *S. uvarum* and the concentration of 4MMP is not linear but suggests that higher proportions of *S. uvarum* result in higher concentrations of 4MMP (Figure 2). Subsequent conditional inference tree analysis using these same four variables resulted in only two terminal nodes which were explained by the proportion of *S. uvarum* at the end of ferment (Figure 3). Ferments with a proportion of *S. uvarum* less than 11 % produced significantly lower concentrations of 4MMP compared to ferments with proportions higher than 11 %.

The effects of species diversity on 3MH and 3MHA

As the production of 3MH and 3MHA are linked, we first investigated whether any variables could explain the molar sum of 3MH and 3MHA. Random forest analysis returned a best model in which the predicted molar sum was negatively correlated with the observed molar sum: meaning we are unable to identify any significant effect on the molar sum of 3MH and 3MHA.

Different species and strains of yeast may affect the equilibrium between 3MH and 3MHA during fermentation (Swiegers et al. 2005a, Coetzee and du Toit 2012). Therefore, we also tested the ratio between 3MH and 3MHA in the wine produced. A random forest analysis explained 7.6 % of the total variance, with geographic region reported as the most important factor (Figure 4). This was followed by the proportion of *S. uvarum* at the end of ferment, then Otu0008 and Otu0053. Taxonomic assignments suggest that Otu0008 corresponds to an unclassified genus of the Sclerotiniaceae family and Otu0053 corresponds to an unclassified species of the Didymellaceae family (Table 1). No significant splits were identified using conditional inference tree analysis.

A closer look at *Saccharomyces* species diversity at the end of ferment

Analyses above show that the proportion of *S. uvarum* at the end of fermentation correlates with thiol concentrations. We performed additional analyses with all 36 samples testing the effect of *Saccharomyces* species composition on thiol concentration. The proportion of *S. uvarum* is significantly positively correlated with the concentration of 4MMP ($r = 0.607$, $t_{34} = 4.46$, $P = 8.55 \times 10^{-5}$). The molar sum of 3MH and 3MHA or the ratio between 3MH and 3MHA are not significantly correlated with the proportion of *S. uvarum* at the end ferment ($r = 0.285$, $t_{34} = 1.74$, $P = 0.092$ and $r = 0.24$, $t_{34} = 1.44$, $P = 0.16$).

DISCUSSION

Microbes are vital for the production of quality agricultural commodities, affecting product quality throughout the development process (Whipps 2001, Fleet 2006, Peiffer et al. 2013, Philippot et al. 2013). For wine we attempt to elucidate which fungal species modulate the production of three important volatile thiols in Sauvignon blanc. We show that different proportions of *Saccharomyces* species driving fermentation, and differences in the fungal community in the starting juice, significantly correlate with thiol concentrations in the wine, recapitulating the importance of microbes in the production of quality agricultural commodities.

Saccharomyces species are responsible for completing fermentation and different species and strains have been shown to produce different metabolites important to the final aroma and flavour of a wine (Masneuf et al. 2002, Howell et al. 2004, Dubourdieu et al. 2006). Here we provide further evidence that higher proportions of *S. uvarum* correlate with higher concentrations of 4MMP (ref?), and this is consistent with reports that *S. uvarum* produces higher concentrations of 4MMP

compared to *S. cerevisiae,* and this difference may be attributed to variation in the IRC7 gene (Masneuf et al. 2002, Dubourdieu et al. 2006, Roncoroni et al. 2011). IRC7 is necessary for 4MMP production by yeast and while it is largely functional in *S. uvarum*, it is often not in *S. cerevisiae* due to a 38 base pair deletion; therefore the presence of a functional IRC7 gene may be responsible for higher levels of 4MMP observed when higher proportions of *S. uvarum* were recorded (Masneuf et al. 2002, Dubourdieu et al. 2006, Roncoroni et al. 2011). This and previous studies show *S. uvarum* is found throughout NZ (Zhang et al. 2010). New Zealand Sauvignon blanc is known for its fruity flavours and high thiol concentrations (Lund et al. 2009). Thus, perhaps the cooler climate combined with the cooler Sauvignon blanc ferment temperatures employed by the NZ wine industry provides *S. uvarum* with an opportunity to more greatly partake in fermentation and thus contribute to higher levels of 4MMP typical of NZ Sauvignon blanc.

The relationship between the community of fungi in juice prior to fermentation and the thiol concentrations in wine is less clear. However, the random forest analyses do show positive correlations between some members of the fungal community and volatile thiol concentrations. While some apparently benign fungal species have been shown to interact with various *S. cerevisiae* strains during fermentation to effect aroma production (Ciani et al. 2006, Viana et al. 2008, Anfang et al. 2009, Comitini et al. 2011), the species of fungi identified in this analysis as potentially effecting thiol concentration in wine are largely associated with plant and fruit diseases. *Alternaria* species on grapes are typically associated with grape bunch rot (Lorenzini and Zapparoli 2014) and the family Sclerotiniaceae contains a range of plant pathogens including *Botrytis cinerea*, which cause noble and bunch rot on grapes. Noble rot can affect the metabolism of the vine itself, altering the composition of the grape berries and thus affecting key aroma and flavour compounds in the

wine (Thibon et al. 2009, Thibon et al. 2011, Blanco-Ulate et al. 2015). Indeed, infection of healthy grapes by *B. cinerea* resulted in wines with higher volatile thiol concentrations, particularly 3MH (Tominaga et al. 2006). Finally, the family Didymellaceae consists of a range of plant pathogens including many representatives of the genus *Phoma*, a known cause of leaf and stem spots (Aveskamp et al. 2008, Zhang et al. 2009). The data presented here suggest that pathogenic fungal species may be able to modulate the flavour and aroma of wine, here specifically thiol concentrations, possibly by their effects on vine and fruit health, and the resulting impact on must composition. This is not surprising as previous research has shown different types of grape rot and disease have different effects on the chemical composition and thus sensorial properties of the wine (Barata et al. 2012). The three volatile thiols examined in these analyses are converted by yeast from odourless precursors in the juice; therefore, if these fungal species affect the accumulation of precursors in the grape they could alter the potential for thiol production during fermentation. Alternatively, these vine and fruit pathogens may directly interact with fermenting yeasts to affect volatile composition in wine. The results presented in this study are by no means conclusive and simply highlight correlations between species abundance and the concentration of three volatile thiols; however, it raises many interesting questions regarding the role of fungal communities throughout the entirety of the grape growing processes and further research into these relationships could prove to be extremely relevant to industry and help inform vineyard management decisions.

It is important to acknowledge that it is possible the correlation of fungal community in the juice and the thiols obtained from wine samples resulted from the differential ripeness of grapes. Aroma compounds accumulate in grapes throughout the ripening process (González-Barreiro et al. 2015),

and if fungal species diversity also changes during fruit ripening, the patterns observed in this analysis may reflect the ripeness of the grapes at harvest, rather than any effects imparted by the fungal community itself. Grape berries have been shown to harbour different fungal communities at different stages of their development with unripe berries reported to have a predominance of *Rhodotorula*, *Cryptococcus* and *Candida* species, along with the yeast-like fungus *Aureobasidium pullulans*, whereas ripe berries additionally harbour *Hanseniaspora* and *Metschnikowia* species (Fleet 2003). Statistical analyses suggest differences in the fungal communities is apparent between grapes at the start of berry ripening and those that are over ripe; however at stages potentially experienced around harvest, no consistent differences in fungal diversity has been detected (Martins et al. 2014). Since all the samples in this study were commercial juices collected at harvest it could be assumed that the fruit was at a similar stage of ripeness and thus the likelihood of this effect confounding our results is not large.

CONCLUSIONS

The results presented here provide further evidence towards the contribution of *S. uvarum* to 4MMP production during fermentation, highlighting the importance of the fermenting population of yeast on the final aroma and flavour of wine. There are also indications that fungi in juice associated with vine and fruit disease may influence the aroma potential of a wine. As this is a correlative study focused on a limited number of volatile thiols, further investigation into the mechanisms of these affects is required; however, these findings do recapitulate the importance of an integrated approach to the study of agricultural phenotypes and quality characteristics and verifies our need for a better understanding of the interaction of microbial ecology in these systems.

References

Anfang, N., M. Brajkovich and M. R. Goddard (2009). Co-fermentation with *Pichia kluyveri* increase varietal thiol concentrations in Sauvignon Blanc. *Australian Journal of Grape and Wine Research* **15**: 1-8.

Aveskamp, M. M., J. De Gruyter and P. W. Crous (2008). Biology and recent developments in the systematics of Phoma, a complex genus of major quarantine significance. *Fungal Diversity* **31**: 1-18.

Barata, A., M. Malfeito-Ferreira and V. Loureiro (2012). The microbial ecology of wine grape berries. *International Journal of Food Microbiology* **153**(3): 243-259.

Benkwitz, F., T. Tominaga, P. A. Kilmartin, C. Lund, M. Wohlers and L. Nicolau (2012). Identifying the chemical composition related to the distinct aroma characteristics of New Zealand Sauvignon blanc wines. *American Journal of Enology and Viticulture* **63**(1): 62-72.

Blanco-Ulate, B., K. C. H. Amrine, T. S. Collins, R. M. Rivero, A. R. Vicente, A. Morales-Cruz, C. L. Doyle, Z. Ye, G. Allen, H. Heymann, S. E. Ebeler and D. Cantu (2015). Developmental and metabolic plasticity of white-skinned grape berries in response to Botrytis cinerea during noble rot. *Plant Physiology* **169**(4): 2422-2443.

Bokulich, N. A., T. S. Collins, C. Masarweh, G. Allen, H. Heymann, S. E. Ebeler and D. A. Mills (2016). Associations among Wine Grape Microbiome, Metabolome, and Fermentation Behavior Suggest Microbial Contribution to Regional Wine Characteristics. *mBio* **7**(3).

Bokulich, N. A., J. H. Thorngate, P. M. Richardson and D. A. Mills (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Sciences of the United States of America* **111**(1): E139-E148.

Breiman, L. (2001). Random Forests. *Machine Learning* **45**: 5-32.

Ciani, M., L. Beco and F. Comitini (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology* **108**(2): 239-245.

Ciani, M., F. Comitini, I. Mannazzu and P. Domizio (2010). Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research* **10**(2): 123-133.

Ciani, M. and F. Maccarelli (1998). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology* **14**(2): 199-203.

Clemente-Jimenez, J. M., L. Mingorance-Cazorla, S. Martínez-Rodríguez, F. J. Las Heras-Vázquez and F. Rodríguez-Vico (2005). Influence of sequential yeast mixtures on wine fermentation. *International Journal of Food Microbiology* **98**(3): 301-308.

Coetzee, C. and W. J. du Toit (2012). A comprehensive review on Sauvignon blanc aroma with a focus on certain positive volatile thiols. *Food Research International* **45**(1): 287-298.

Comitini, F., M. Gobbi, P. Domizio, C. Romani, L. Lencioni, I. Mannazzu and M. Ciani (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiology* **28**(5): 873-882.

Cutler, D. R., T. C. Edwards, K. H. Beard, A. Cutler, K. T. Hess, J. Gibson and J. J. Lawler (2007). Random forests for classification in ecology. *Ecology* **88**(11): 2783-2792.

Darriet, P., T. Tominaga, V. Lavigne, J.-N. Boidron and D. Dubourdieu (1995). Identification of a powerful aromatic component of *Vitis vinifera* L. var. sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour and Fragrance Journal* **10**(6): 385-392.

de Melo Pereira, G. V., C. L. Ramos, C. Galvão, E. Souza Dias and R. F. Schwan (2010). Use of specific PCR primers to identify three important industrial species of *Saccharomyces* genus: *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces pastorianus*. *Letters in Applied Microbiology* **51**(2): 131-137.

Demuyter, C., M. Lollier, J. L. Legras and C. Le Jeune (2004). Predominance of *Saccharomyces uvarum* during spontaneous alcoholic fermentation, for three consecutive years, in an Alsatian winery. *Journal of Applied Microbiology* **97**(6): 1140-1148.

Dubourdieu, D., T. Tominaga, I. Masneuf, C. P. Des Gachons and M. L. Murat (2006). The role of yeasts in grape flavor development during fermentation: The example of Sauvignon blanc. *American Journal of Enology and Viticulture* **57**(1): 81-88.

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince and R. Knight (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**(16): 2194-2200.

Fleet, G. H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology* **86**(1-2): 11-22.

Fleet, G. H. (2006). The commercial and community significane of yeasts in food and beverage production. The yeast handbook volume 2: Yeasts in food and beverages. A. Querol and G. H. Fleet. Germany, Springer-Verlag Berlin Heidelberg**:** 1-12.

Gobbi, M., F. Comitini, P. Domizio, C. Romani, L. Lencioni, I. Mannazzu and M. Ciani (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology* **33**(2): 271-281.

Goddard, M. R. (2008). Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecology* **89**(8): 2077-2082.

González-Barreiro, C., R. Rial-Otero, B. Cancho-Grande and J. Simal-Gándara (2015). Wine aroma compounds in grapes: A critical review. *Critical Reviews in Food Science and Nutrition* **55**(2): 202-218.

Harsch, M. J., F. Benkwitz, A. Frost, B. Colonna-Ceccaldi, R. C. Gardner and J.-M. Salmon (2013). New Precursor of 3-Mercaptohexan-1-ol in Grape Juice: Thiol-Forming Potential and Kinetics during Early Stages of Must Fermentation. *Journal of Agricultural and Food Chemistry* **61**(15): 3703-3713.

Hastie, T., R. Tibshirani and J. H. Friedman (2009). The elements of statistical learning: data mining, inference, and prediction. New York, Springer.

Hernández-Orte, P., M. Cersosimo, N. Loscos, J. Cacho, E. Garcia-Moruno and V. Ferreira (2008). The development of varietal aroma from non-floral grapes by yeasts of different genera. *Food Chemistry* **107**(3): 1064-1077.

Horthorn, T., K. Hornik and A. Zeileis (2006). Unbiased recursive partioning: A conditional inference framework. *Journal of Computational and Graphical Statistics* **15**(3): 651-674.

Howell, K. S., D. Cozzolino, E. J. Bartowsky, G. H. Fleet and P. A. Henschke (2006). Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. *FEMS Yeast Research* **6**(1): 91-101.

Howell, K. S., J. H. Swiegers, G. M. Elsey, T. E. Siebert, E. J. Bartowsky, G. H. Fleet, I. S. Pretorius and M. A. de Barros Lopes (2004). Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains. *FEMS Microbiology Letters* **240**(2): 125-129.

Knight, S. and M. R. Goddard (2015). Quantifying separation and similarity in a *Saccharomyces cerevisiae* metapopulation. *ISME Journal* **9**: 361-370.

Knight, S., S. Klaere, B. Fedrizzi and M. R. Goddard (2015). Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to terroir. *Scientific Reports* **5**: 14233.

Kurtzman, C. P. and C. J. Robnett (2003). Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. *FEMS Yeast Research* **3**(4): 417-432.

Lambrechts, M. G. and I. S. Pretorius (2000). Yeast and its importance to wine aroma - a review. *South African Journal of Enology and Viticulture* **21**: 97-129.

Liaw, A. and M. Wiener (2002). Classification and regression by randomForest. *R News* **2**(3): 18-22.

Lorenzini, M. and G. Zapparoli (2014). Characterization and pathogenicity of Alternaria spp. strains associated with grape bunch rot during post-harvest withering. *International Journal of Food Microbiology* **186**(0): 1-5.

Lund, C. M., M. K. Thompson, F. Benkwitz, M. W. Wohler, C. M. Triggs, R. Gardner, H. Heymann and L. Nicolau (2009). New Zealand Sauvignon blanc distinct flavor characteristics: Sensory, chemical, and consumer aspects. *American Journal of Enology and Viticulture* **60**(1): 1-12.

Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y.-J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. I. Alenquer, T. P. Jarvie, K. B. Jirage, J.-B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley and J. M. Rothberg (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**(7057): 376-380.

Martins, G., J. Vallance, A. Mercier, W. Albertin, P. Stamatopoulos, P. Rey, A. Lonvaud and I. Masneuf-Pomarède (2014). Influence of the farming system on the epiphytic

yeasts and yeast-like fungi colonizing grape berries during the ripening process. *International Journal of Food Microbiology* **177**: 21-28.

Masneuf-Pomarède, I., M. Bely, P. Marullo, A. Lonvaud-Funel and D. Dubourdieu (2010). Reassessment of phenotypic traits for *Saccharomyces bayanus* var. *uvarum* wine yeast strains. *International Journal of Food Microbiology* **139**(1–2): 79-86.

Masneuf-Pomarède, I., C. Mansour, M. L. Murat, T. Tominaga and D. Dubourdieu (2006). Influence of fermentation temperature on volatile thiols concentrations in Sauvignon blanc wines. *International Journal of Food Microbiology* **108**(3): 385-390.

Masneuf, I., M. L. Murat, G. I. Naumov, T. Tominaga and D. Dubourdieu (2002). Hybrids *Saccharomyces cerevisiae* x *Saccharomyces bayanus* var. *uvarum* having a high liberating ability of some sulfur varietal aromas of *Vitis vinifera* Sauvignon Blanc wines. *Journal International des Sciences de la Vigne et du Vin* **36**(4): 205-212.

McMurdie, P. J. and S. Holmes (2014). Waste not, want not: Why rarefying microbiome data is inadmissible. *PLoS Comput Biol* **10**(4): e1003531.

Mortimer, R. and M. Polsinelli (1999). On the origins of wine yeast. *Research in Microbiology* **150**(3): 199-204.

Murat, M. L., I. Masneuf, P. Darriet, V. Lavigne, T. Tominaga and D. Dubourdieu (2001). Effect of *Saccharomyces cerevisiae* yeast strains on the liberation of volatile thiols in Sauvignon blanc wine. *American Journal of Enology and Viticulture* **52**(2): 136-139.

Naumov, G. I., I. Masneuf, E. S. Naumova, M. Aigle and D. Dubourdieu (2000). Association of *Saccharomyces bayanus* var. *uvarum* with some French wines: genetic analysis of yeast populations. *Research in Microbiology* **151**(8): 683-691.

Naumova, E. S., G. I. Naumov, I. Masneuf-Pomarède, M. Aigle and D. Dubourdieu (2005). Molecular genetic study of introgression between Saccharomyces bayanus and S. cerevisiae. *Yeast* **22**(14): 1099-1115.

New Zealand Winegrowers (2016). "Annual report 2016." Retrieved 29 March 2017, 2017, from http://www.nzwine.com/assets/sm/upload/4n/2f/ux/9c/NZW_Annual_Report%202016_WEB.pdf.

Peiffer, J. A., A. Spor, O. Koren, Z. Jin, S. G. Tringe, J. L. Dangl, E. S. Buckler and R. E. Ley (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences of the United States of America* **110**(16): 6548-6553.

Philippot, L., J. M. Raaijmakers, P. Lemanceau and W. H. van der Putten (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology* **11**(11): 789-799.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16**(8): 675-729.

Quince, C., A. Lanzen, T. P. Curtis, R. J. Davenport, N. Hall, I. M. Head, L. F. Read and W. T. Sloan (2009). Accurate determination of microbial diversity from 454 pyrosequencing data. *Nature Methods* **6**(9): 639-641.

Romanelli, A. M., D. A. Sutton, E. H. Thompson, M. G. Rinaldi and B. L. Wickes (2010). Sequence-based identification of filamentous Basidiomycetous fungi from clinical specimens: a cautionary note. *Journal of Clinical Microbiology* **48**(3): 741-752.

Romano, P., C. Fiore, M. Paraggio, M. Caruso and A. Capece (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology* **86**(1-2): 169-180.

Romano, P., L. Granchi, M. Caruso, G. Borra, G. Palla, C. Fiore, D. Ganucci, A. Caligiani and V. Brandolini (2003a). The species-specific ratios of 2,3-butanediol and acetoin isomers as a tool to evaluate wine yeast performance. *International Journal of Food Microbiology* **86**(1-2): 163-168.

Roncoroni, M., M. Santiago, D. O. Hooks, S. Moroney, M. J. Harsch, S. A. Lee, K. D. Richards, L. Nicolau and R. C. Gardner (2011). The yeast IRC7 gene encodes a β-lyase responsible for production of the varietal thiol 4-mercapto-4-methylpentan-2-one in wine. *Food Microbiology* **28**(5): 926-935.

Rueckert, A. and H. W. Morgan (2007). Removal of contaminating DNA from polymerase chain reaction using ethidium monoazide. *Journal of Microbiological Methods* **68**: 596-600.

Sadoudi, M., R. Tourdot-Maréchal, S. Rousseaux, D. Steyer, J. J. Gallardo-Chacón, J. Ballester, S. Vichi, R. Guérin-Schneider, J. Caixach and H. Alexandre (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiology*.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn and C. F. Weber (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**(23): 7537-7541.

Sicard, D. and J. L. Legras (2011). Bread, beer and wine: Yeast domestication in the Saccharomyces sensu stricto complex. *Comptes Rendus - Biologies* **334**(3): 229-236.

Swiegers, J. H., E. J. Bartowsky, P. A. Henschke and I. S. Pretorius (2005a). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research* **11**(2): 139-173.

Swiegers, J. H., R. L. Kievit, T. Siebert, K. A. Lattey, B. R. Bramley, I. L. Francis, E. S. King and I. S. Pretorius (2009). The influence of yeast on the aroma of Sauvignon Blanc wine. *Food Microbiology* **26**(2): 204-211.

Swiegers, J. H. and I. S. Pretorius (2005). Yeast modulation of wine flavor. Advances in Applied Microbiology. J. W. B. Allen I. Laskin and M. G. Geoffrey, Academic Press. **57:** 131-175.

Swiegers, J. H., R. Willmott, A. Hill-Ling, D. L. Capone, K. H. Pardon, G. M. Elsey, K. S. Howell, M. A. de Barros Lopes, M. A. Sefton, M. Lilly and I. S. Pretorius (2006). Modulation of volatile thiol and ester aromas by modified wine yeast. Developments in Food Science. L. P. B. Wender and P. Mikael Agerlin, Elsevier. **Volume 43:** 113-116.

Taylor, M. W., P. Tsai, N. Anfang, H. A. Ross and M. R. Goddard (2014). Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environmental Microbiology* **16**(9): 2848-2858.

Thibon, C., S. Cluzet, J. M. Mérillon, P. Darriet and D. Dubourdieu (2011). 3-Sulfanylhexanol Precursor Biogenesis in Grapevine Cells: The Stimulating Effect of Botrytis cinerea. *Journal of Agricultural and Food Chemistry* **59**(4): 1344-1351.

Thibon, C., D. Dubourdieu, P. Darriet and T. Tominaga (2009). Impact of noble rot on the aroma precursor of 3-sulfanylhexanol content in *Vitis vinifera* L. cv Sauvignon blanc and Semillon grape juice. *Food Chemistry* **114**(4): 1359-1364.

Tominaga, T., Y. Niclass, E. Frérot and D. Dubourdieu (2006). Stereoisomeric distribution of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate in dry and sweet white wines made from Vitis vinifera (Var. Sauvignon blanc and Semillon). *Journal of Agricultural and Food Chemistry* **54**(19): 7251-7255.

Tominaga, T., C. Peyrot des Gachons and D. Dubourdieu (1998a). A new type of flavor precursors in *Vitis vinifera* L. cv. Sauvignon Blanc: S-cysteine conjugates. *Journal of Agricultural and Food Chemistry* **46**(12): 5215-5219.

Torriani, S., G. Zapparoli and G. Suzzi (1999). Genetic and phenotypic diversity of *Saccharomyces* sensu stricto strains isolated from Amarone wine. *Antonie van Leeuwenhoek* **75**(3): 207-215.

Viana, F., J. V. Gil, S. Genovés, S. Vallés and P. Manzanares (2008). Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiology* **25**(6): 778-785.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* **52**(suppl 1): 487-511.

Xufre, A., H. Albergaria, J. Inácio, I. Spencer-Martins and F. Gírio (2006). Application of fluorescence in situ hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *International Journal of Food Microbiology* **108**(3): 376-384.

Zhang, H. Y., S. A. Lee, J. E. Bradbury, R. N. Warren, H. Sheth, D. O. Hooks, K. D. Richards and R. C. Gardner (2010). Yeasts isolated from New Zealand vineyards and wineries. *Aust J Grape Wine R* **16**: 491-496.

Zhang, Y., C. L. Schoch, J. Fournier, P. W. Crous, J. de Gruyter, J. H. C. Woudenberg, K. Hirayama, K. Tanaka, S. B. Pointing, J. W. Spatafora and K. D. Hyde (2009). Multi-locus phylogeny of Pleosporales: a taxonomic, ecological and evolutionary re-evaluation. *Studies in Mycology* **64**(0): 85-102.

Zott, K., C. Thibon, M. Bely, A. Lonvaud-Funel, D. Dubourdieu and I. Masneuf-Pomarede (2011). The grape must non-Saccharomyces microbial community: Impact on volatile thiol release. *International Journal of Food Microbiology* **151**(2): 210-215.

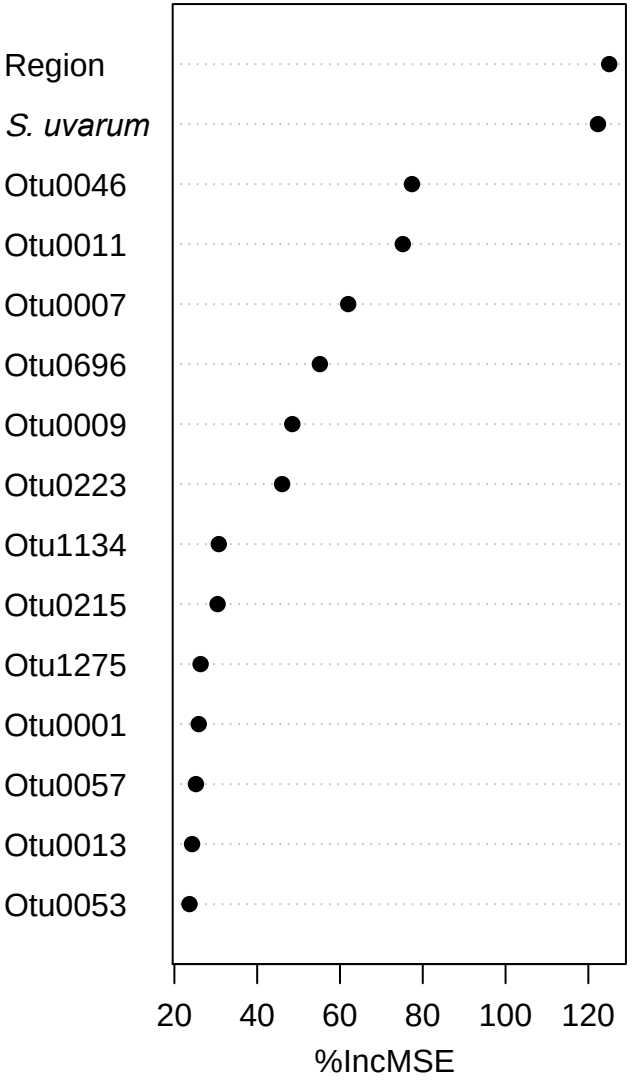
Figure Legends

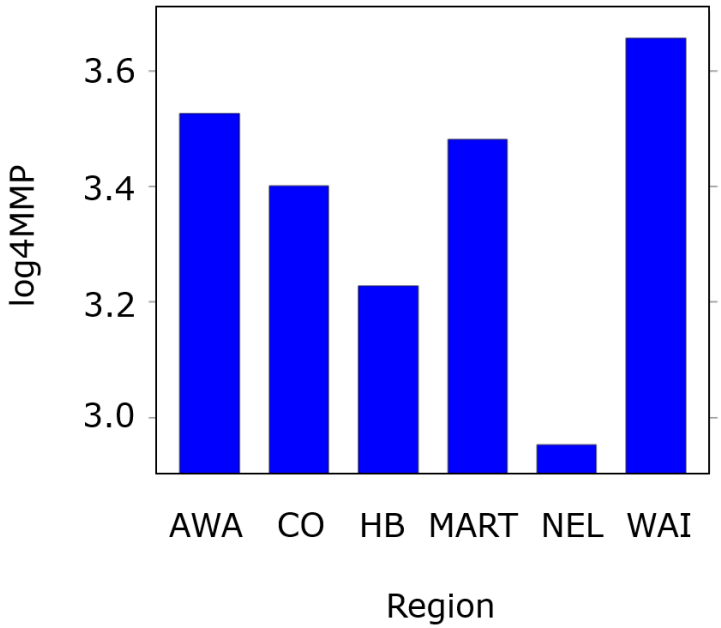
Figure 1: Variable importance as a measure of the percent increase in mean square error when that variable is removed from the random forests analysis for 4MMP. The taxonomic identification of the OTUs are listed in Table 1.

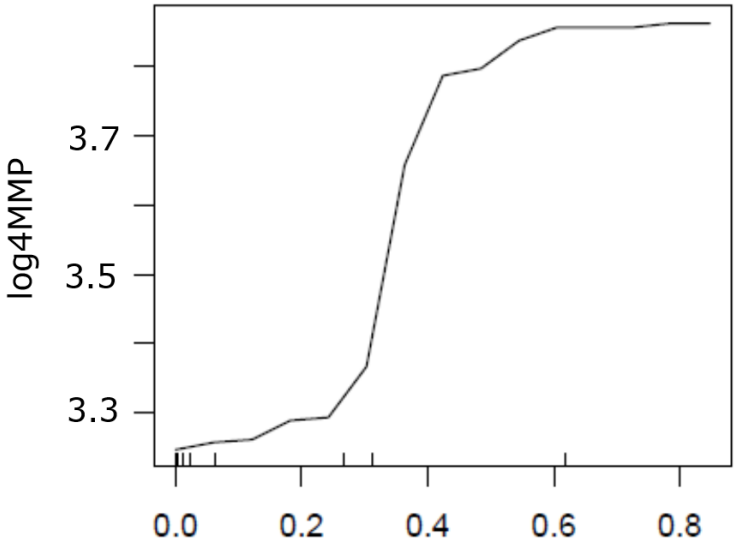
Figure 2: 4MMP partial dependence plots for the top four variables identified in the random forest analysis while holding the other variables constant. The tick marks on the x-axis of the graphs represent the deciles of the training data and thus reflect the spread of the data. The abbreviations for each region are as follows: HB, Hawke's Bay; NEL, Nelson; CO, Central Otago; MART, Martinborough; WAI, Wairau Valley; AWA, Awatere Valley. The location of each of the regions is shown in Sup. Figure 1.

Figure 3: Conditional inference tree explaining the concentration of 4MMP, performed using the 25 wine samples in Dataset S2. Only three nodes were identified in this analysis, with the proportion of *S. uvarum* (Sacch) at the end of ferment being identified as the only explanatory variable that significantly affects 4MMP concentration. The 18 wine samples with a proportion of *S. uvarum* at the end of ferment less than 0.277 shown in the left box plot have significantly lower concentrations of 4MMP compared to the 7 wine samples with a proportion of *S. uvarum* higher than 0.277 shown in the right box plot ($P = 0.002$).

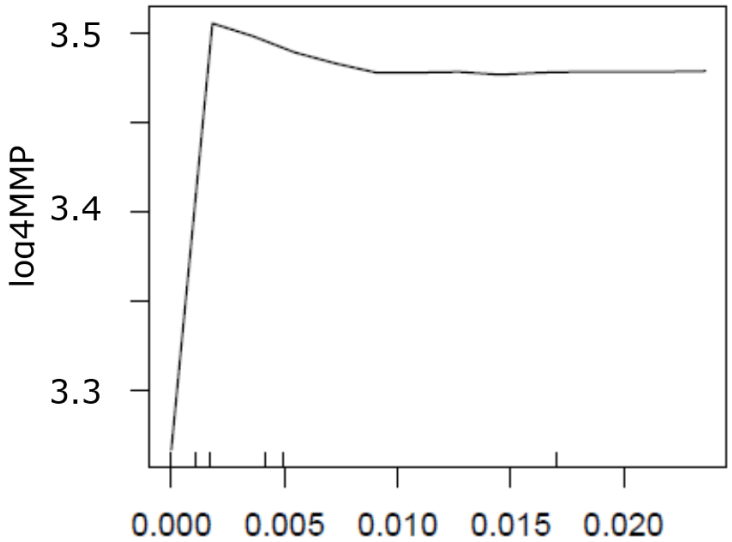
Figure 4: Variable importance as a measure of the percent increase in mean square error when that variable is removed from the random forests analysis for the ratio between 3MH and 3MHA. The taxonomic identification of the OTUs are listed in Table 1.


Region
S. uvarum
Otu0046
Otu0011
Otu0007
Otu0696
Otu0009
Otu0223
Otu1134
Otu0215
Otu1275
Otu0001
Otu0057
Otu0013
Otu0053
20
40
60
80
100
120
%IncMSE

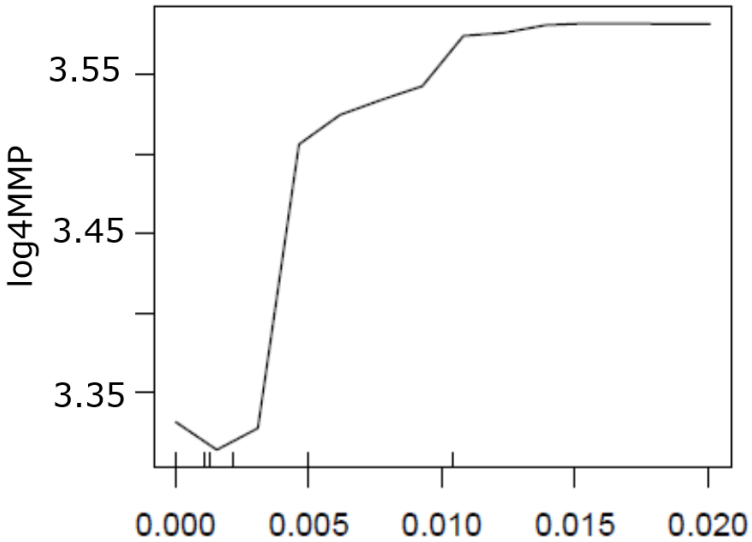




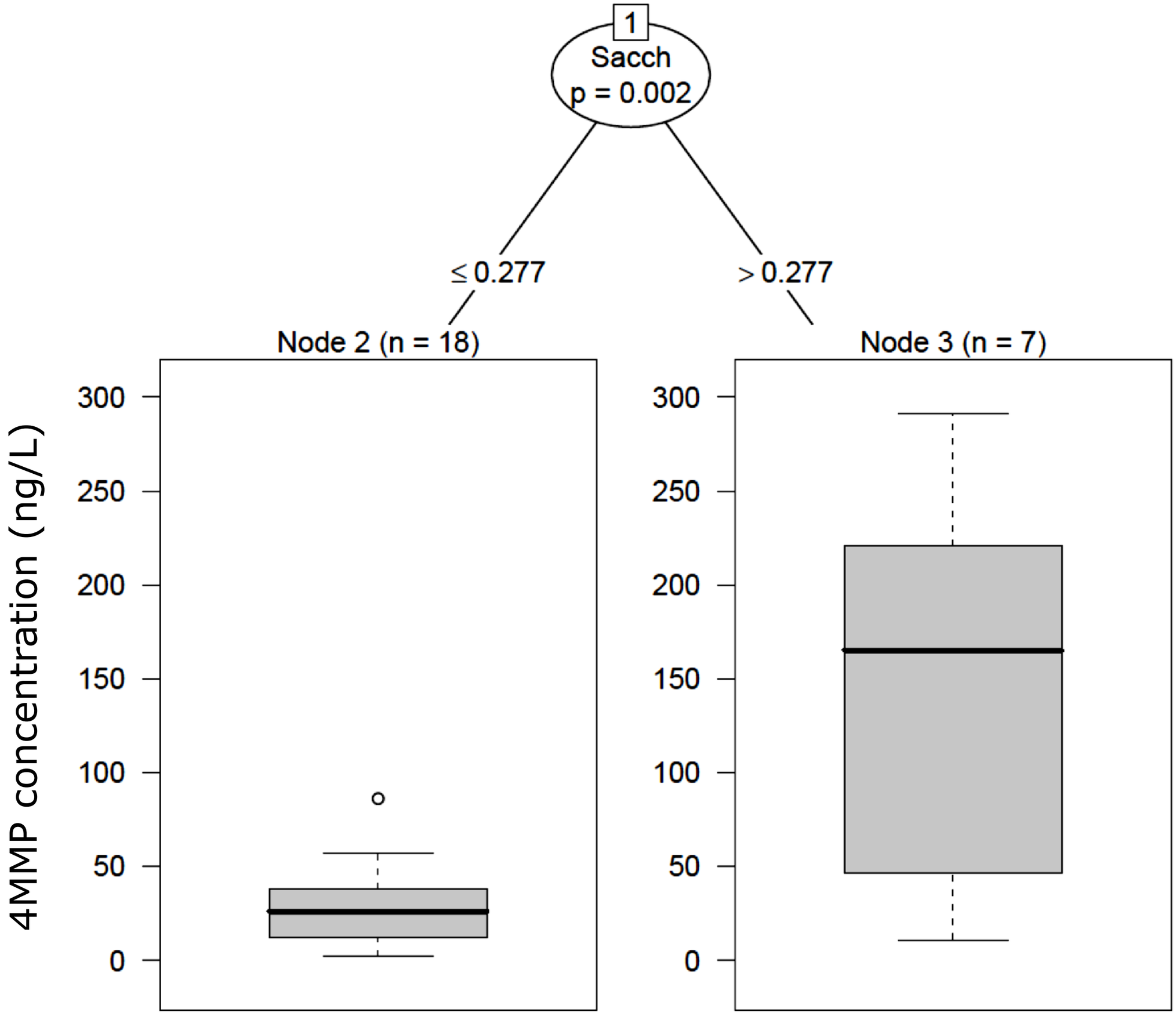
Proportion of *S. uvarum* at the end of ferment



Proportion of Otu0046: *Penicillium* sp.



Proportion of Otu0011: *Alternaria* sp.


1
Sacch
p = 0.002
≤ 0.277
> 0.277
Node 2 (n = 18)
Node 3 (n = 7)
4MMP concentration (ng/L)
300
250
200
150
100
50
0

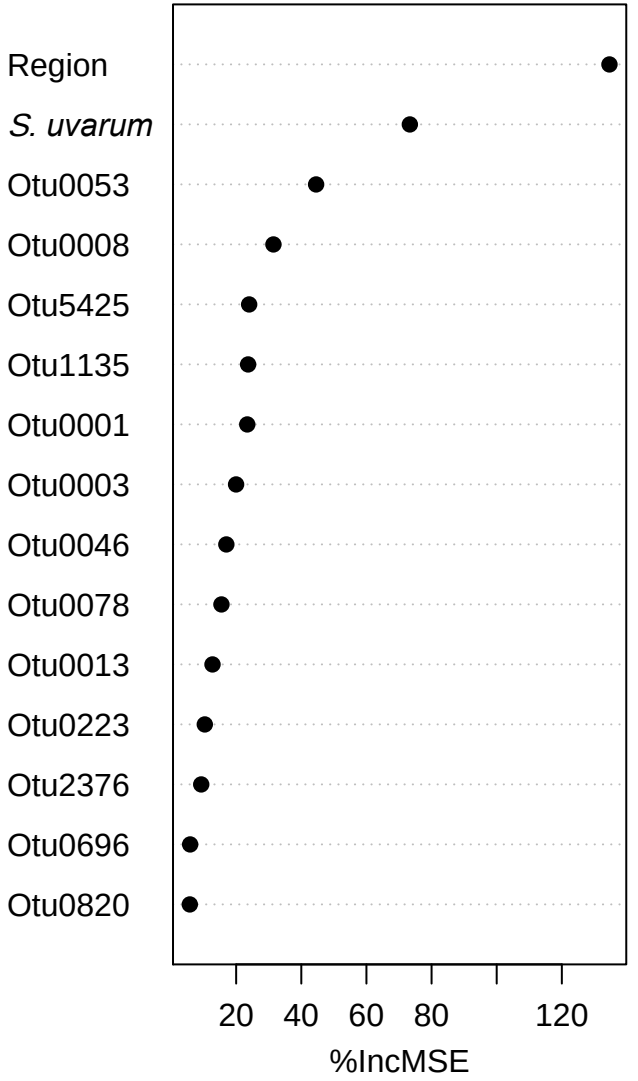

Region
S. uvarum
Otu0053
Otu0008
Otu5425
Otu1135
Otu0001
Otu0003
Otu0046
Otu0078
Otu0013
Otu0223
Otu2376
Otu0696
Otu0820
20
40
60
80
120
%IncMSE

Table 1. The taxonomic identification of the OTUs identified and presented in the random forest analyses

OTU code	Genus identification
Otu0001	*Columnosphaeria*
Otu0003	*Cladosporium*
Otu0007	*Torulaspora*
Otu0008	Unclassified (Family: Sclerotiniaceae)
Otu0009	*Davidiella*
Otu0011	*Alternaria*
Otu0013	*Hanseniaspora*
Otu0046	*Penicillium*
Otu0053	Unclassified (Family: Didymellaceae)
Otu0057	*Columnosphaeria*
Otu0078	*Wickerhamomyces*
Otu0215	Unclassified (Phylum: Ascomycota)
Otu0223	Unclassified (Kindom: Fungi)
Otu0696	Unclassified (Phylum: Ascomycota)
Otu0820	Unclassified (Phylum: Ascomycota)
Otu1134	Unclassified (Order: Dothideales)
Otu1135	*Phaeodothis*
Otu1275	Unclassified (Phylum: Ascomycota)
Otu2376	*Malassezia*
Otu5425	Unclassified (Phylum: Ascomycota)